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CULTURE OF MYCOBACTERIA

The present invention relates to a method of culture of mycobacteria, to a growth medium therefor, and to a method of culture of mycobacteriophage.

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Health risks associated with mycobacteria have been known for many years and diseases caused by some species are responsible for major global healthcare problems.

- 10 The emergence of antibiotic resistant strains of Mycobacteria responsible for tuberculosis (TB) have led to increasing numbers of deaths in those contracting the infection. A TB vaccine based on an attenuated strain of *M.bovis* (BCG) has been available for several years, but protection is restricted to particular ethnic groups, for reasons that are unknown. Mycobacteria have also been associated
- 15 with several other conditions such as Crohn's Disease.

In addition, there are indications that inoculation with products derived from some mycobacterium species can mediate changes in immune responses which have beneficial effects. Thus, products of this type are currently undergoing evaluation

20 to investigate their therapeutic usefulness against a range of conditions, including TB and cancers.

- A major problem associated with the study and production of pharmaceutical products based on mycobacteria is the difficulty associated with bacterial growth.
- 25 Conventional methods involve growth on solid agar slopes and, consequently, manufacturing products using this type of approach is both labour intensive and costly. These processes are poorly defined leading to batch variation.

Development of improved culture processes have made only limited progress with

30 problems of relatively slow growth rate and bacterial aggregation. Batch culture processes have been associated with apparent loss of virulence, and/or essential components.

The majority of studies to date have grown *M.tuberculosis* as surface pellicles or as agitated dispersed cultures over long periods. Media used have been relatively simple containing a carbon source, nitrogen and buffered salts together with trace elements and were designed to provide high yields free of macromolecular medium components. Glycerol is considered the essential carbon source to ensure copious growth.

Media for culture of tubercle bacilli is described by Dupos *et al* in AM.REV.TUBERC. volume 56, 1947, pp334-345. A growth medium referred to as "Tween®-albumin" medium was used, containing 0.01-0.05 percent Tween® 80 and from 0.5-1.0 percent albumin. This growth medium has hitherto been the standard growth medium used in this field. Wayne L.G. in Infection and Immunity, Sept.1977, pp528-530 used this same medium and reported a mean generation time of 17-18 hours for *Mycobacterium tuberculosis*. Lowrie *et al*, in Journal of General Microbiology, Volume 110, 1979, pp431-441 used a concentrated version of the same medium. A review by Wayne L.G in Tuberculosis: Pathogenesis, Protection, and Control, published by the American Society for Microbiology in 1994, pp73-83 also describes how most published work in this area has employed 0.02 percent Tween® 80 and 0.5 percent bovine serum albumin, the albumin being used to protect the bacillus from toxic effects of traces of oleate released from the Tween® 80. Youmans and Youmans reported in 1959 that 0.05 per cent Tween® 80 slowed mycobacterial growth noticeably.

Another problem with existing culture methods and culture media is that the mean generation time, or doubling time, of the bacillus is rather long. For commercial production of mycobacteria it would be desirable to reduce the doubling time so that a greater yield of bacillus and bacillus products, such as enzymes, may be obtained. Typically, it is found that a culture period of at least two or three weeks is necessary before the culture may be harvested to yield any useable volume of products.

Wayne L.G, who has published widely in this area, has reported that growth of

mycobacteria in detergent-containing medium results in diminution of virulence, which is a serious disadvantage.

5 It is also found that the existing culture methods of mycobacteria produce rather low yields. Lowrie *et al* obtained yields of around 8×10^8 bacteria ml^{-1} , but only at very low dilution rates of 0.016h^{-1} , indicating a doubling time of about 43 hours. Wayne describes a culture having a quicker doubling time, but only at cell densities in the region of 4×10^7 CFU ml^{-1} . It would accordingly be desirable to provide a culture having a reduced doubling time whilst maintaining high bacterial
10 densities.

It is desirable to produce mycobacteria at increased yields. Whilst the maintainance of bacterial virulence is desirable for the purpose of vaccine preparations, the production of avirulent bacteria possessing virulent surface
15 epitopes is also desirable for vaccine preparation. High yields of BCG mycobacteria would be particularly useful in manufacture of the BCG vaccine. Currently, vaccine components are made using many hundreds or even thousands of individual flasks. This is highly inefficient, with batch to batch variation, but continuous culture methods or large scale fermenter culture are not
20 available to replace this inefficient method.

An objective of the present invention is to provide for batch or continuous culture of mycobacteria, in particular continuous culture that will maintain bacterial virulence (or avirulent bacteria possessing virulent cell surface epitopes), and
25 provide cells of defined and consistent properties. Another objective is to provide a growth medium for culture of mycobacteria.

As mentioned above, conventional culture methods have achieved relatively poor yields of mycobacteria. This, in turn, has meant that high yield
30 mycobacteriophage culture methods have proved problematic. In this respect, conventional solid phase preparation methods inherently produce limited phage yields, whereas conventional liquid phase yields are dependent on *inter alia* the

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concentration of mycobacterial host available in the culture medium. Thus, a further objective of the present invention is to provide a method of culturing mycobacteriophage which overcomes/alleviates the prior art poor yield problems.

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Accordingly, a first aspect of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch fermenter culture or continuous culture, with agitation and in the presence of at least 0.1% (v/v) detergent. Sufficient detergent is present so that a substantially homogenous suspension of cells is maintained.

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Preferably, the method of the invention comprises growing said mycobacteria in batch fermenter culture or continuous culture, at a temperature of 35°C +/- 10°C, at a dissolved oxygen tension of at least 1.0 percent, at a pH of 6.9 +/- 0.9.

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In use of the present invention, illustrated by specific embodiments described below in more detail, and using *Mycobacteria tuberculosis*, we have developed a method which allows high yields of bacteria from both batch and continuous culture systems. Further, we have shown that mycobacteria generated using the methods of embodiments of the present inventions are highly virulent as demonstrated in a standard guinea p-g infection model of *M.tuberculosis*. Indeed, potency of *Mycobacteria tuberculosis* grown using these methods is comparable with *M.tuberculosis* grown using the solid agar slope method.

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In a specific embodiment of the present invention, growth of *M.tuberculosis* in steady-state continuous culture achieved a biomass yield of 1.2gl⁻¹ cell dry weight. Cells grown in this continuous culture, and also in batch culture, displayed virulence comparable to cells grown on Middlebrook agar slopes, strongly indicating the suitability of these methods for growth of mycobacteria spp, such as *M. tuberculosis* or *M. bovis*, for prolonged periods in chemostat culture.

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It is thus an advantage that the method of the invention enables growth at

increased cell densities and with reduced mean generation times, or doubling times. It is further of advantage that using the method of the present invention expression of virulence determinants has been maintained. Thus, the method is of application for production of mycobacteria such as for incorporation into BCG vaccines.

In the present invention, the term "batch culture" is used in its conventional sense to refer to a fixed volume of culture medium which is inoculated with a micro-organism. After a period of adjustment, termed the lag phase, the organism starts to grow and multiply reaching the maximum growth rate possible in that environment – termed exponential growth. After multiple generations essential nutrients become depleted or toxic metabolites build-up causing growth to slow and eventually cease. This is a closed system and the environment is constantly changing as the organism grows. This type of culture is typically performed in shake flasks, 50–500 ml, where only temperature is controlled though in embodiments of the invention temperature, pH and oxygen have been controlled. A further benefit of methods of the invention is that through control of environmental parameters there is reduced batch-to-batch variation, leading to cultures of more consistent composition and less bacterial heterogeneity, which is a significant consideration during production of vaccine components from these cultures.

The term "fermenter culture" is similarly used with reference to a type of batch culture operated with more control over the environmental parameters such as pH and aeration. Fermenters are normally used for production, hence the culture volume is larger.

The term "continuous culture" is used to refer typically to a culture of constant volume to which medium is added continuously and from which there is continuous removal of any overflow culture. By adding growth components in the fresh medium, the organism continues to multiply. When this system reaches equilibrium, cell number and nutrient status remain constant, it is said to be in

steady state.

5 Lastly, the term "chemostat culture" refers to the current most common type of continuous culture device. Two elements are generally used to control the culture, the concentration of an essential nutrient, such as carbon source, and the flow rate. After inoculation the culture grows until an essential nutrient becomes depleted and limits growth, however, the continuous addition of fresh medium containing the limiting nutrient permits continued growth. The cell density is controlled by the concentration of limiting nutrient added. The limiting nutrient can be altered by manipulating the medium formulation. The rate of medium addition controls the growth rate (generation time) of the culture.

15 In the method of the invention, it is preferred that the culture temperature is maintained at $35^{\circ}\text{C} \pm 10^{\circ}\text{C}$, more preferably $35^{\circ}\text{C} \pm 5^{\circ}\text{C}$, and in specific embodiments of the invention this preferred temperature has been maintained for in excess of three weeks with continuous mycobacteria growth. The pH of the culture medium, in continuous operation, is typically controlled to within ± 0.9 of pH 6.9, more preferably ± 0.5 of pH 6.9. pH may be controlled using addition of acid or alkaline solution to the culture medium, according to the pH correction required. In specific embodiments of the invention described below, sodium hydroxide at a concentration of 0.5M and sulphuric acid at a concentration of 0.5M is used. The dissolved oxygen concentration of the culture is typically at an initial level of at least 40% (v/v) air saturation, preferably at least 50%.

25 Detergent is present in the method of the invention as a dispersing agent to maintain a high proportion of the mycobacteria suspended in a substantially homogenous suspension, preferably as single cells or small clumps containing 2 to 10 bacilli, preferably 2 to 5 bacilli. In one embodiment, at least 50%, preferably at least 75%, particularly preferably at least 90% of the total mycobacterial cell weight is suspended as above.

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Once thus dispersed, cells can grow in an environment enabling higher growth

rates under relatively constant and controlled conditions. It is possible, though the applicant does not wish to be bound by any theory, that once mycobacteria form pellicles as in previous culture methods they can not thereafter be dispersed - the invention may thus improve the previous methods by preventing or reducing loss of bacilli into such pellicles. Some detergents in use slowly release toxic components into the culture medium, so the amount of detergent present should not be so high as to risk the detergent or any of its components reaching toxic levels. Similarly, excess detergent can lead to foaming of the culture and should be avoided. The level of detergent may suitably be at least 0.1% (v/v).

Anionic detergents are preferred, in particular esters of sorbitan and derivatives thereof, though it is believed that the advantageous effects of the invention and the results obtained in the specific embodiments may likewise be realised using any of a wide range of detergents. Particularly good results have been obtained using a polyethane-diyl derivative of a sorbitan ester, namely Tween® 80, other such esters being Tween® 20, Tween® 40 and Tween® 60. Despite the presence of detergent it has been found that albumin may be omitted from the growth medium without slowing mycobacterial growth.

In use of the methods of the invention for batch culture of mycobacteria, detergent may be present at from 0.1 to 1.0 % (v/v), more preferably from 0.1 to 0.5 %, most preferably about 0.2 % (v/v).

In use of the methods of the invention for continuous culture of mycobacteria, detergent may be present at least 0.1 % (v/v), more preferably at least 0.15 % (v/v), and most preferably about 0.2%, its level further preferably being no more than 1.0%, and usually no more than 0.7%. When the culture is being operated continuously, medium is continuously introduced into the culture, the rate of introduction expressed as a dilution rate. The culture of the invention can be carried out continuously with a dilution rate of at least 0.02 h^{-1} , resulting in a high yield of bacteria, and these bacteria have been found to have preserved their virulence. A dilution rate of at least 0.025 h^{-1} can also be sustained, and in a

specific embodiment a dilution rate of about 0.03 h^{-1} was achieved in continuous culture, representing a mean doubling time of about 24 hours.

The invention further provides, in a second aspect, a growth medium for culture of mycobacteria, comprising:-

- a carbon source;
- a mitogen;
- trace elements comprising at least Mg, K, P and S;
- a nitrogen source; and
- at least 0.1% (v/v) detergent.

The carbon source is preferably selected from glucose, glycerol and an amino acid, and combinations of these carbon sources. The mitogen is present to induce cell division and is preferably asparagine, though other mitogens from inorganic sources are also suitable. Trace elements in the growth medium are preferably selected from Ca, Mg, Zn, Co, Cu, Mn, Fe, K and mixtures thereof, and the nitrogen source is selected from an amino acid and an ammonium salt.

The growth medium optionally further comprises an amino acid component selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, serine, and mixtures thereof. The amino acid component can contribute the nitrogen source in the medium.

Other optional components are a vitamin/co-factor component selected from:- inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thioctic acid, and mixtures thereof, preferably biotin; and one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and α -ketoglutarate, preferably glycerol and/or pyruvate.

Thus, a particularly preferred embodiment of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch fermenter culture or continuous culture, with agitation in the presence of at least 0.1% (v/v) detergent so that a substantially homogenous suspension of single cells is maintained, and in the

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presence of a growth medium according to combination of the above-described media.

The mycobacterial culture methods and media of the present invention are suitable for culture of all members of the *Mycobacteria tuberculosis* complex (MTC) as well as mutant and recombinant forms thereof. In one embodiment, the methods and media are used for culture of *M. tuberculosis*, but are also suitable for culture of *M. bovis* and other opportunistic mycobacteria.

10 According to a third aspect of the present invention there is provided a method of culture of mycobacteriophage, comprising culture of mycobacteria as described above, and contacting said mycobacteria with a mycobacteriophage. In a preferred embodiment, the phage may be added directly to the mycobacterial liquid culture.

15 Reference to mycobacteriophage includes mutant and recombinant forms thereof.

A mycobacteriophage is any phage which is capable of infecting and replicating in a mycobacterium. The mycobacteriophage need not be specific for the mycobacterium which it infects. However, it may be preferred that the phage exhibits specificity for a given mycobacterial species or even sub-species.

20 In one embodiment the phage culture method is employed to culture a phage capable of infecting *M. tuberculosis*, *M. bovis* and/or *M. paratuberculosis*.

25 The phage culture method is particularly suited to the culture of phage capable of infecting, and which are preferably specific for, *M. tuberculosis*.

30 In specific embodiments of the present invention, the phage to be cultured is selected from the group consisting of D-34 (Accession No. ATCC 4243-B1), LG (Accession No. ATCC 25618-B1), DS6A (Accession No. ATCC 25618-B2), and D29 (Froman *et al.* 1954).

The preferred mycobacteriophages for use in this aspect of the invention are phages which are capable of causing a lytic infection. This facilitates downstream phage harvesting.

- 5 According to the phage culture method, native or genetically engineered or chemically modified bacteriophage which require susceptible mycobacteria for growth can be generated effectively.

- 10 Thus, mycobacteriophage or component parts or phage nucleic acid having prophylactic or therapeutic use, and which may also be used as gene delivery systems in whole or in part, may be grown and manufactured in quantities suitable for clinical and commercial application.

- 15 Growth of mycobacteria is performed in a controlled culture system using the culture medium described.

To facilitate phage propagation, the medium may be modified by the incorporation of an agent which is capable of promoting and/or assisting phage adsorption on the mycobacteria cell surface.

- 20 Preferred agents include bovine serum albumin and other molecules having cell surface adsorption-promoting properties such as cations. In use, the latter are typically employed at a concentration of approximately 0.015 M.

- 25 In one embodiment an agent is incorporated at a final concentration of between 0.01 and 1% w/v, preferably between 0.05% and 0.5% w/v. A typical final concentration is approximately 0.1% w/v.

- 30 The agent is preferably added to the mycobacteria culture medium prior to or substantially at the same time as inoculation of the mycobacteriophage.

Since the mycobacteria culture medium of the present invention includes a

detergent, it is preferable to reduce or minimise exposure of the inoculating phage to the detergent because the presence of detergent may have a deleterious effect on phage propagation, possibly through interference with phage adsorption on the mycobacterial cell wall. As mentioned above, the use of an agent may help alleviate potential detergent-related problems.

To this end, a number of method steps are provided for reducing/minimising exposure of the inoculating phage to detergent present in the culture medium. These include:-

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(i) reducing the concentration of detergent prior to phage inoculation. This may be achieved by, for example, arresting agitation of the mycobacteria culture medium to allow the mycobacteria to settle by gravity, whereafter detergent-containing medium may be removed from towards the top of the culture vessel and replaced by detergent-free fresh medium;

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(ii) as an alternative to (i) fresh detergent-free medium may be added to the culture vessel to dilute the overall detergent concentration; or

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(iii) as in (i) above, wherein settling of the bacteria is accelerated by use of a flocculating agent. Following removal of detergent-containing medium and replacement with detergent-free fresh medium, an anti-flocculating agent may be added to reverse/neutralise the effects of the flocculating agent.

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In a preferred embodiment, subsequent to phage inoculation and following a sufficient time period to allow phage infection to be established, the detergent concentration of the medium may be increased, for example to substantially the same concentration as prior to phage inoculation, thereby providing optimal mycobacterial growth conditions once again.

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For phage propagation, the infectious phage seed culture (ie. inoculum) is typically prepared from seed bank stocks stored at high tighter suspension (eg.

10^9 - 10^{10} pfu/ml⁻¹) in, for example, phosphate buffer saline solution at -20°C.

5 Infectious phage seed culture may take the form of purified, semi-purified phage, or a mixture of phage and phage-infected mycobacteria which has been generated in, for example, shake flasks or smaller culture vessels.

10 The time of phage seed inoculation may vary according to the mycobacterium, medium, phage, and the multiplicity of infectious dose (MOI) employed. Typically, the MOI is in the range of one phage to 10 mycobacteria, but may be varied in accordance with the system employed.

15 Phage inoculation may occur at any point during the mycobacterial growth cycle. Preferably, inoculation occurs approximately 25-35 hours following initiation of bacterial logarithmic growth. Typically, inoculation occurs 30 hours following initiation of bacterial logarithmic growth.

Culture conditions are preferably monitored and maintained for a further 24 hour period to allow the desired number of bacteriophage replication cycles to occur.

20 Purification of phage may be achieved using conventional chromatography methods, such as immunoaffinity purification for phage which has been engineered to express an appropriate ligand, or centrifugation of phage from filtered culture supernatant followed by resuspension in an appropriate buffer.

25 In some instances, infected cells which have yet to undergo phage-induced lysis may be harvested through conventional filtration methods and lysed mechanically or chemically or by ultrasound to release contained phage which may then be further purified as described above.

30 The present invention is now described with reference to the following specific embodiments, illustrated by drawings in which:-

Fig. 1 shows a schematic view of continuous culture apparatus according to the invention;

Fig. 2 shows a graph of optical density at 540 before and after initiation of continuous culture;

Fig. 3 shows viable *M. tuberculosis* in guinea pig lungs following aerosol challenge with bacteria grown using the medium of the invention; and

Fig. 4 shows viable *M. tuberculosis* in guinea pig spleens following aerosol challenge with bacteria grown using the medium of the invention.

Referring to Fig. 1, a medium reservoir 1 is attached via medium addition pump and line 2 to culture vessel 6. The glass culture vessel 6 comprises a titanium top plate through which are connected temperature probe 7, oxygen electrode 8, air inlet and sparger 9, vent 10, pH electrode 11, alkali addition line 12 and acid addition line 13. Samples of the content of the culture vessel may be taken through sample port 14 and effluent from the culture vessel drains into or is pumped into effluent reservoir 15. The remaining features in Fig. 1 are: a magnetic stirrer unit 3; a heating pad 4; and a magnetic bar 5.

This continuous culture apparatus is used for continuous culture of mycobacteria as described in examples below.

Fig. 2 illustrates the continuous culture of *M. tuberculosis*. After inoculation, the culture was operated in batch for 4 days. Medium addition was then initiated in fed-batch mode. Continuous medium addition was started at 300 h.

Fig. 3 illustrates the viable *M. tuberculosis* in guinea pig lungs following aerosol challenge (error bars + standard deviation are shown) and compares the influence of culture mode on the virulence of *M. tuberculosis*. The virulence of chemostat grown cells was compared with cells grown to mid-exponential batch

phase in ABCD ModTB medium and on Middlebrook agar. Guinea pig challenge with plate-grown cells produced a classical disease process with exponential multiplication in guinea pig lungs up to three weeks post-infection, when lung counts reached 10^6 to 10^7 c.f.u. per lung. After 3 weeks the lung counts declined marginally. Low numbers of bacilli were detected in spleen tissues 2 weeks post-infection followed by an exponential increase up to day 21. Infection with both batch and chemostat grown cells produced a comparable disease process demonstrating that culture virulence was retained.

Fig. 4 illustrates viable *M. tuberculosis* in guinea pig spleens following aerosol challenge as in Fig. 3 (error bars + standard deviation are shown).

Example 1

Materials and Methods

Strain

Studies was performed with *M. tuberculosis* strain H37Rv (NCTC cat. no. 7416) - a representative strain of *M.tuberculosis*. Stock cultures were grown on Middlebrook 7H10 + OADC for 3 weeks at $37 \pm 2^\circ\text{C}$ harvested and stored at -70°C as a dense suspension in deionised water.

Culture Medium

A chemically defined culture medium was developed, and was designated CAMR Mycobacterial Medium (see Appendix 1 below). The medium was prepared with high quality water from a Millepore water purification system and filter sterilised by passage through a $0.07 \mu\text{m}$ pore size cellulose acetate membrane filter capsule (Sartorius Ltd). Middlebrook 7H10 + OADC agar was used to prepare inoculum cultures, enumerate the number of culturable bacteria in chemostat samples, and to assess culture purity.

Culture apparatus

Culture experiments were performed in a one litre glass vessel operated at a

working volume of 500 ml. The culture was agitated by a magnetic bar placed in the culture vessel coupled to a magnetic stirrer positioned beneath the vessel. Culture conditions were continuously monitored and controlled by an Anglicon Microlab Fermentation System (Brighton Systems, Newhaven), linked to sensor probes inserted into the culture through sealed ports in the top plate. The oxygen concentration was monitored with a galvanic oxygen electrode (Uniprobe, Cardiff) and was controlled through feedback control of the agitation rate. Temperature was monitored by an Anglicon temperature probe, and maintained by a heating pad positioned beneath the culture vessel. Culture pH was measured using an Ingold pH electrode (Mettler-Toledo, Leicester) and controlled by automatic addition of either sodium hydroxide (0.5 M) or sulphuric acid (0.5 M). For continuous culture, the culture system was operated by controlling nutrient addition from the medium reservoir and a constant culture volume was maintained by an overflow tube fitted to the side of the vessel.

Inoculation and culture

The vessel was filled with 350 ml of sterile culture medium and parameters were allowed to stabilise at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $\text{pH } 6.9 \pm 0.2$ and a dissolved oxygen tension of approximately 70% air saturation. A dense inoculum suspension was prepared by resuspending Middlebrook agar cultures, grown at $37 \pm 2^{\circ}\text{C}$ for 3 week, in sterile deionised water. The inoculum was aseptically transferred to the culture vessel, to provide an initial culture turbidity of approximately 0.25 at 540 nm. After inoculation the culture was allowed to establish for approximately 50 h. As the culture entered exponential growth, a further 100 ml medium was added and batch growth was monitored by optical density and viable count determination.

For continuous culture, the culture was inoculated and allowed to establish for approximately 50 h as detailed. The culture was then operated in fed batch mode for 48 h with medium addition (approx. 100 ml) as the culture entered exponential growth and 24 h later. Continuous culture was then initiated at a dilution rate of 0.03 h^{-1} [equivalent to a mean generation time (MGT) of 24 h]. Culture parameters were maintained at a dissolved oxygen tension of 50 % (v/v) air

saturation at $37 \pm 2^\circ\text{C}$ and $\text{pH } 6.9 \pm 0.2$. Growth was monitored by optical density, dry weight and viable count determination.

Culture analyses

- 5 The optical density of culture samples was recorded at 540 nm (OD_{540}) in a UV-260 spectrophotometer (Pye Unicam) against a water reference. Culture biomass was determined by dry weight analysis. Samples were treated with 4% (v/v) formaldehyde for at least 24 h and filtered through a pre-dried, pre-weighed, 0.45 μm pore sized, nylon membrane filter (Gelman Sciences), under vacuum. The
10 membrane was rinsed with 10 ml of deionised water, before re-drying to a constant weight, and re-weighing.

- Total viable counts were performed by preparing a 10-fold dilution series of the sample in sterile deionised water, and plating 100 μl aliquots of appropriate
15 dilutions onto Middlebrook 7H10 plates in triplicate. The plates were incubated at 37°C for 3 weeks before enumerating the number of colonies formed. Culture purity was checked by plating neat samples onto Middlebrook 7H10 and Blood agar and incubating at 37°C .

20 Results

Batch culture

- Growth of *M. tuberculosis* strain H37Rv was established in CAMR Mycobacteria Medium supplemented with 0.2% Tween[®] 80. After inoculation the culture followed typical batch growth kinetics with a lag phase of approximately 50 hours
25 before entering exponential growth. A minimum doubling time of approximately 14 h was recorded. Cultures were predominantly single cell suspensions.

Continuous culture

- Steady-state growth, at a MGT of 24 h, was normally reached 10 days after
30 initiation of continuous culture. Cultures were dense suspensions containing approximately $5 \times 10^8 \text{ cfu ml}^{-1}$ and a biomass yield of approximately 1.2 g l^{-1} cell dry weight. Cells were short rods 2 to 3 μm long with occasional clumps of up to

20 cells. Glycerol, the principal carbon source was not depleted during steady state growth, with a residual concentration of 1.25 g l^{-1} . Tween® 80 was present in an amount of 0.1% and enabled the growth of *M. tuberculosis* in a homogeneous suspension made up substantially of single cells at a growth rate conducive to chemostat culture. Cultures grown in the absence of Tween® 80 formed large clumps and surface pellicles and continuous culture was not possible.

Other observations made during operation of this culture indicate that for long-term continuous production it may be necessary to clean the vessel or preferably transfer to a clean vessel at regular intervals, say every 5-6 weeks. Mycobacteria can sometimes tend to attach to the vessel wall, impeding continuous culture, and we have also found, separately, that lowering the oxygen tension to at least 20% air saturation assists to counter this problem.

Influence of culture mode on virulence

The virulence of batch and chemostat grown cells was compared with cells grown on Middlebrook agar. Guinea pigs challenged with plate-grown cells produced a classical disease process with exponential multiplication in guinea pig lungs up to three weeks post infection, when lung counts reached 10^6 to 10^7 c.f.u. per lung (fig. 3). Low numbers of bacilli were detected in spleen tissues 2 weeks post-infection followed by an exponential increase up to day 21, after which growth rate declined (fig. 4). Infection with both batch and chemostat grown cells produced a comparable disease process demonstrating that culture virulence was retained.

The invention thus provides methods for batch and continuous culture of dispersed mycobacteria in high yield and without loss of virulence, and also provides a growth medium therefor. In cultures of the invention, large-scale and consistent production of vaccine components is enabled, for manufacture eg. of bacterial subunits, whole bacilli for vaccine uses and whole bacilli for immune therapies.

Example 2**Production of Mycobacteriophage****Bacteriophage:**

- 5 Mycobacteriophage e.g. D-34 (Accession No. ATCC 4243-B1) is specific for *M. tuberculosis*. Stock bacteriophage is prepared from liquid culture or soft agar overlay and is stored as a high titre suspension 10^9 - 10^{10} pfu ml⁻¹ in PBS at - 20°C.
- 10 **Growth of *M. tuberculosis* and bacteriophage propagation:**
Growth of *M. tuberculosis* strain H37Rv is performed in the controlled culture system using the CAMR Mycobacterium Medium as detailed previously in Example 1.
- 15 For phage propagation, the CAMR Mycobacterium Medium is modified by the incorporation of 0.1% (w/v) bovine serum albumin (BSA). After inoculation, the culture is allowed to establish and turbidity at 540 nm is monitored to determine the onset of exponential growth. Stock bacteriophage suspension is slowly thawed and added to the culture 30 h after the initiation of logarithmic growth.
- 20 A multiplicity of infection of 1 phage to 10 bacilli is used. Culture conditions are continuously monitored and maintained for a further 24 h or until two cycles of phage propagation have occurred. Bacteriophage replication can be followed by monitoring the change in culture turbidity and oxygen utilisation.
- 25 The culture is pelleted by centrifugation at 10,000 g for 15 min and the supernatant containing the bacteriophage is retained. The bacteriophage is concentrated by ultrafiltration, washed with phosphate buffered saline and filter sterilized by passage through a 0.45µm cellulose acetate membrane filter.
- 30 The titre of the concentrated bacteriophage suspension is determined against *M. tuberculosis* using the conventional soft overlay method.

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